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Effect of a pre-calving injectable trace mineral supplement on white blood cell function in seasonally calving pastoral dairy cows

AJ Bates [a,b], M Wells [c], C Fitzpatrick [d] and RA Laven [b]

[a]Vetlife NZ, Vetlife Scientific, Temuka, New Zealand; [b]Tāwharau Ora – School of Veterinary Science, Massey University, Palmerston North, New Zealand; [c]Virbac New Zealand Ltd., Hamilton, New Zealand; [d]Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand

ABSTRACT

Aims: To investigate the effect of injection of trace mineral supplement (TMS) 14–28 days before calving on white blood cell count (WBCC) and function, serum antioxidant capacity (SAC) and reactive oxygen species (ROS) in pasture-fed cattle after calving.

Methods: On each of two South Island, seasonally calving, pastoral dairy farms,1 month before dry-off, a random sample of 150 multiparous cows predicted to calve within 7 days of the herd's planned start of calving (PSC) were stratified on individual somatic cell count, age, breed and expected calving date. On each farm, 14–24 days before PSC, 60 selected cows were randomly assigned for TMS (Zn, Mn, Se, Cu) injection, and 60 were controls. All 240 cows were contemporaneously injected with hydroxocobalamin, and controls with Se. Blood samples were collected pre-injection and 3, 12 and 40 days after calving. Phagocytic activity, count and proportion of neutrophils, lymphocytes and monocytes, WBCC, ROS, SAC were measured. Plasma concentrations of Se, Cu and glutathione peroxidase (GPx) were monitored from a random subset of animals. Differences attributable to TMS were estimated using mixed-multivariable Bayesian analysis, expressed as mean and highest density interval (HDI).

Results: Three and 40 days after calving, TMS-treated cows had 0.36 (90% HDI = 0.00–0.77) x 10^9 and 0.25 (90% HDI = 0.00–0.55) x 10^9 fewer neutrophils/L. Neutrophils comprised 6 (90% HDI = 0–11)% and 4 (90% HDI = 0–8)% less of the WBCC, and the neutrophil count was 14 (90% HDI = 0–27)% and 9 (90% HDI = 0–18)% less than controls. However, 3 days after calving, there were 7 (95% HDI = 2–12)% more cells phagocytosing and 2,900 (95% HDI = 2,600–3,200) more bacteria ingested/cell. Twelve and 40 days after calving, TMS-treated cows had 0.65 (95% HDI = 0.17–1.17) x 10^9 and 0.28 (95% HDI = 0.00–0.59) x 10^9 more lymphocytes/L. Lymphocytes comprised 10 (95% HDI = 3–18)% and 5 (95% HDI = 0–9)% more of the WBCC, and the lymphocyte count was 30 (95% HDI = 11–51)% and 9 (95% HDI = 0–9)% more than controls. There were no meaningful differences in ROS, SAC, ROS/SAC, other white blood cells, or WBCC. Plasma Cu, Se and GPx concentrations were above recommended thresholds.

Conclusions: Pre-calving TMS injection was associated with differences in white blood cell population and function that may reduce the risk of disease.

Abbreviations: BHOB: Beta-hydroxybutyrate; GPx: Glutathione peroxidase; HDI: Highest density interval; MESF: Molecules of equivalent soluble fluorophore; OSi: Oxidative stress index; PSC: Planned start of calving; ROPE: Region of probable equivalence; ROS: Reactive oxygen species; SAC: Serum antioxidant capacity; THI: Temperature humidity index; TMS: Trace mineral supplement; WAIC: Widely applicable information criterion; WBCC: White blood cell count.

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Trace mineral supplementation; phagocytosis; leukocyte; oxidative stress; periparturient immunity

Introduction

A collection of interrelated challenges contributes to the increase in periparturient disease seen in cows as they adapt to the demands of lactation (Drackley *et al.* 2005) and multiple cow and herd level risk factors have been identified in seasonal, pastoral systems (Bates and Dohoo 2016; Bates and Saldias 2017).

In periparturient dairy cows in all systems, there is an increase in immune challenge from greater production of endogenous reactive oxygen species (ROS; Sordillo 2016) and a depletion of serum antioxidant capacity (SAC; LeBlanc 2020). This leads to an imbalance of oxidation–reduction homeostasis (or oxidative stress) with an increase in damage to tissues and decreased immune function (De Vliegher *et al.* 2012; Sordillo 2016; LeBlanc 2020). This has led Abuelo *et al.* (2013) to suggest that the ratio of ROS/SAC is indicative of an oxidative stress index (OSi) that reflects the animal's oxidative status.

CONTACT AJ Bates a.bates@massey.ac.nz
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Immune function is also compromised as mobilisation of body energy reserves and lipolysis around calving lead to insulin resistance and uncoupling of the somatotropic axis (Baumgard *et al.* 2017). Together with decreases in dry matter intake and negative energy balance in early lactation (Vazquez and Smith 2000) there is disruption of the homeorhetic regulation of the inflammatory component of the immune response (Thompson-Crispi and Mallard 2012) and alterations in inflammatory response and immune activation (De Vliegher *et al.* 2012; Horst *et al.* 2021). Pathological disruptions of these systems are known co-contributors to periparturient disease in dairy cows (Godden *et al.* 2003; Aleri *et al.* 2016; LeBlanc 2020).

Trace mineral supplementation (TMS) may have a role in countering some of these effects. TMS containing Zn, Se, Mn and Cu, which are all components of antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase, has been linked to improvements in oxidative status in poultry (Abd El-Hack *et al.* 2017; Rajkumar *et al.* 2018) and, under conditions of heat stress, sheep (Alhidary *et al.* 2015) and dairy cattle (Silva *et al.* 2022). An injectable form of TMS containing these minerals may also boost immune activation in dairy animals, potentially countering some of the effects of the negative energy balance. Silva *et al.* (2022) reported improvements in white blood cell function, and inflammatory status after calving in cows under conditions of heat stress following injection of TMS containing Zn, Mn, Se and Cu pre-partum. Injectable TMS containing Zn, Mn, Se, Cu and Cr, given to calves at birth, was also associated with an increase in the proportion of neutrophils and monocytes showing phagocytosis and more bacteria ingested per cell (Bates *et al.* 2020). Similar results were reported by Teixeira *et al.* (2014) where injection of a TMS containing Zn, Mn, Se and Cu at 3 and 30 days after birth increased neutrophil function and GPx activity in the first 50 days of life. Improvements in the humoral and cellular immune response following injection of TMS containing Zn, Se and Cu have also been reported in 3.5-month-old calves when given an attenuated live bacterin vaccine (Bittar *et al.* 2018) or a modified live viral vaccine (Palomares *et al.* 2016).

However, the clinical effects from injection of TMS on adult dairy cows appear to be variable across studies. Machado *et al.* (2013) reported reductions in periparturient endometritis and clinical and subclinical mastitis following TMS containing Zn, Mn, Se and Cu pre-calving, while Bates *et al.* (2022) reported reductions in periparturient clinical and subclinical mastitis but found no effect on endometritis when using a similar product. However, the diagnosed prevalence of endometritis is highly dependent on the method of detection (Dubuc *et al.* 2010; Wagener *et al.* 2017; McDougall *et al.* 2020), which differed between these two studies. Despite the improvements in immune function reported above, Silva *et al.* (2022) found no effect in their study on periparturient mastitis. However, there was a reduction in OR for metritis although the upper boundary of the 95% CI reached the null value (OR = 0.68, 95% CI = 0.4–1.0, p = 0.051).

There is thus some variability in the clinical response to TMS injection and none of the published studies reporting an effect of TMS on immune function or status in adult cows have taken place under comparable conditions to those seen in New Zealand's spring calving, pastoral herds. Further, although in the summer, dairy cows across New Zealand are exposed to conditions where they may experience heat stress, the risk of this during spring calving is considerably reduced even though there is some evidence that the combination of a pastoral diet, high levels of ambient ultra-violet radiation and large distances walked may increase their susceptibility (Bryant *et al.* 2007).

Against this variable clinical response and uncertainty over the presence of potential contributing factors identified in other studies, this study was designed as a follow-up to Bates *et al.* (2022) to investigate the effect of injectable TMS containing Zn, Mn, Se and Cu, 24–28 days before calving on the OSi, white blood cell function and population of white blood cells over the periparturient period in pastorally farmed dairy cows in New Zealand.

Materials and methods

All procedures were approved by the Massey University Animal Welfare Ethics Committee, reference 22/22.

Enrolment

The trial was conducted between May 2022 and September 2022 on two commercial, seasonally calving pastoral dairy farms with predominantly Friesian herds serviced by Vetlife Ltd. (Timaru, NZ). The schedule of trial events is shown in Figure 1. Farms were selected as a convenience sample from those serviced by Vetlife, that agreed to participate, and that had a rotary milking platform with automatic drafting facilities. As a condition of enrolment, both farms vaccinated multiparous milking cows with a pre-calving anti-scour vaccination 2–4 weeks before calving (Rotavec Corona; Schering-Plough Animal Health Ltd, Upper Hutt, NZ).

To establish that concentrations of serum Cu, Se and plasma GPx were within reference ranges, in May 2022, 14–28 days from the end of lactation (Visit 1, Figure 1), a coccygeal blood sample was collected from 15 enrolled cows on each farm into vacuum tubes with sodium heparin anticoagulant and separately into tubes with EDTA (Vacutainer; BD Diagnosis, Auckland, NZ). Cows were randomly selected by

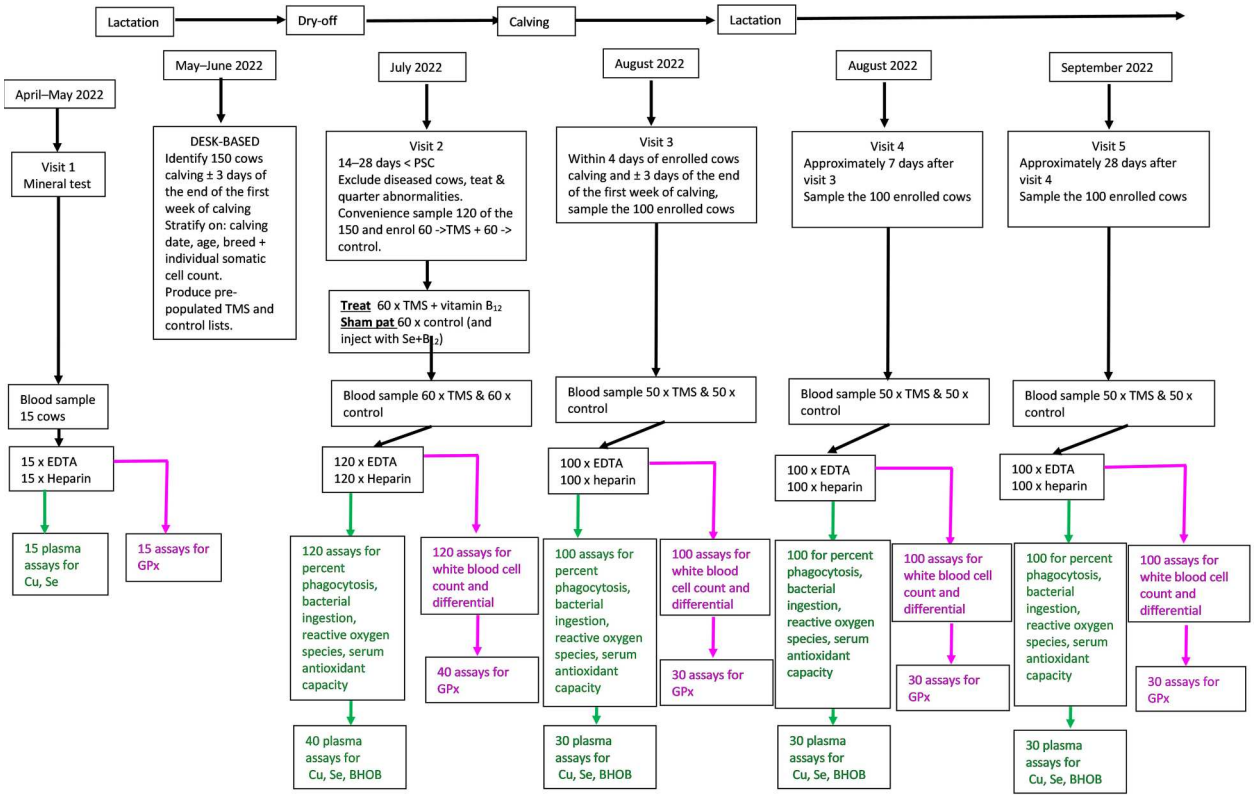

Figure 1. Timeline summary of key events relative to the planned start of calving (PSC) within a trial investigating the effect of a pre-calving injectable trace mineral supplement (TMS) on white blood cell function and population, plasma Cu, Se, glutathione peroxidase (GPx) and beta-hydroxybutyrate (BHOB) in seasonally calving pastoral dairy cows in New Zealand.

sampling every n/15th cow where n was the number of cows currently in milk in the herd. Samples were couriered to a commercial veterinary laboratory (Gribbles Veterinary, Christchurch, NZ) within 24 hours and analysed for GPx activity and Cu and Se concentrations in plasma. Samples were refrigerated overnight at 1–4°C if necessary.

Management and nutrition

Over the lactation preceding the trial (August 2021 – May 2022) all milking cows were fed a predominantly ryegrass (*Lolium perenne*) pasture, with supplementary milled wheat (*Triticum aestivum*) at 1–2 kg/cow/day and ryegrass silage depending on stocking rate and pasture supply. Throughout lactation, all cows were on water supply supplemented with a customised blend of minerals (AgVance Nutrition; Auckland, NZ) providing an estimated daily dose per cow of 10 mg Co as cobalt sulphate, 80 mg Cu as copper glycinate, 137.5 mg Cu as copper sulphate, 16.0 mg I as ethylenediamine dihydroiodide, 5.8 mg Se as selenium selenite, and 450 mg Zn as zinc sulphate.

All cows were dried off over the same 2-week period at the end of May 2022. Dry-off policy varied between farms but was the same on each farm for all enrolled cows and followed standard New Zealand management guidelines (DairyNZ 2013, 2020). All dry cows were wintered on fodder beet (*Beta vulgaris*) without further mineral supplementation, transitioning to a grass-based diet 2–3 weeks before calving and returning to the mineralised water supply with an additional 60 g magnesium chloride, 60 g magnesium oxide and 50 g dicalcium phosphate per cow per day mixed into perennial ryegrass silage.

Sample frame

On each farm, the target population was cows without grossly abnormal teat-ends, signs of clinical disease (reduced ruminal fill, abdominal pain, foul smelling diarrhoea, swelling or redness in the udder), or non-functional quarters, and that were expected to calve within the first week of calving as measured from the planned start of calving (PSC).

After drying off, cows with suitable predicted calving dates were identified on each farm using records held on Minda, the national Livestock Improvement Corporation (Hamilton, NZ) database. Up to 150 of these cows (300 across both farms) were randomly selected into a dedicated Excel database (Microsoft Corp., Redmond, WA, USA) using the RAND function within this program and stratified into treatment and control groups on predicted calving date, age, and individual somatic cell count at the last herd test for that lactation and breed.

At 14–28 days from the herd's PSC (Visit 2, Figure 1) and coincident with the injection of anti-calf-scour

vaccine, these cows were drafted at the milking shed for potential administration of TMS or control treatment. As cows entered the milking platform, a Vetlife veterinarian using an aerosol paint spray (Tell Tail Aerosol; GEA-FIL, Mt Maunganui, NZ) identified any cows with teat abnormalities, signs of clinical disease or non-functional quarters. These cows were excluded from the study. Once the milking platform was full (n = 50–60, depending on the farm), the sample frame was populated by selecting the first 60 cows (as they presented on the milking platform) that had been identified as pre-assigned to the TMS group and the first 60 cows pre-identified as assigned to the control group. Thus, the sample population was a convenience sample drawn from a random sample of cows meeting the eligibility criteria. Power calculations (see Supplementary Information) indicated that 50 cows were required per group, but an additional 10 cows per group were enrolled pre-calving to allow for losses to follow-up between enrolment and the first post-calving sampling occasion.

Experimental groups

Cows allocated to the treatment group received a SC injection at 1 mL/100 kg body weight of TMS containing 40 mg Zn, 10 mg Mn, 5 mg Se and 15 mg Cu per mL (Multimin; Virbac NZ Ltd., Hamilton, NZ). Body weights were estimated on an individual basis by eye and rounded upwards to the nearest 100 kg to approximate conditions that are commonly employed on-farm. Historically on both farms, 2–4 weeks before calving, all cows were routinely injected SC with 1 mL/100 kg of a short-acting preparation of Se and vitamin B_{12} providing 5 mg Se and 2,000 µg hydroxocobalamin/mL. Both farms wanted to retain supplementation and so cows allocated to the TMS group also received 1 mL/100 kg SC of vitamin B_{12} (Prolaject B_{12} 2000; Elanco Animal Health, Auckland, NZ), to provide the same dose of vitamin B_{12} without doubling up on the dose of Se. Control cows were injected SC with 1 mL/100 kg of Se and vitamin B_{12} (Prolaject B_{12} 2000 Plus Selenium; Elanco Animal Health) and manually patted at an equivalent site to mimic the additional TMS injection.

Contemporaneously, coccygeal blood samples were collected separately into heparin and EDTA-lined vacutainers from all 60 control and 60 TMS-injected cows on each farm. All personnel were subsequently blinded to treatment status. After calving, follow-up coccygeal blood samples were collected within 4 days of calving from the first 50 TMS and 50 control cows that calved within the first week of the herd's PSC (Visit 3, Figure 1). The same cows were blood sampled again 12 and 40 days after calving (Visit 4 and 5 in Figure 1, respectively).

Laboratory procedures: on-farm Visits 2–5

The tests selected to measure the immune capability and environment required a plasma sample collected with a heparin anticoagulant, while the assay for white blood cell count (WBCC) and differential required a whole blood sample collected using EDTA-lined vacutainers. To minimise the number of blood tubes collected per cow, plasma Cu, Se and beta-hydroxybutyrate (BHOB) were assayed using the heparin tubes. After collection, the heparinised blood was stored in an insulated box at ambient temperature to preserve the phagocytic and oxidative burst capacity (Teixeira *et al.* 2014) and samples containing EDTA were placed on ice.

Analysis of white blood cell function and oxidative status

Heparin samples were transported within 4 hours to the University of Otago (Dunedin, NZ) for assessment of neutrophil and monocyte function, SAC and ROS.

Neutrophil and monocyte phagocytic activity were assessed using the PHAGOTEST kit (Orpegen Pharma GmbH, Heidelberg, Germany) containing fluorescein-labelled opsonised *Escherichia coli* following the manufacturer's instructions. Cells were analysed using a FACS-Calibur flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) using a 488-nm argon-ion laser. Ten thousand events were collected for each cell population (neutrophils and monocytes) and the results were reported as percentage of the total number of cells in the granulocyte gate performing phagocytosis, and as the mean increase of the green fluorescence of the gated cells expressed as molecules of equivalent soluble fluorophore (MESF) (Schwartz *et al.* 2002) and corresponding to the number of ingested bacteria per cell (Teixeira *et al.* 2014).

Subsequently, the heparin samples were centrifuged at 2,000 g for 3 minutes and assayed for ROS as described by (Trotti *et al.* 2001) using the spectrophotometric d-ROM test (Diacron International, Grosseto, Italy), which determines hydroperoxides (breakdown products of lipids as well as of other organic substrate, generated by the oxidative attack of ROS) through their reaction with the chromogen N,N-diethylparaphenylenediamine. Results were expressed in arbitrary "Carratelli Units" (CarrU), where 1 CarrU is equivalent to the oxidising power of 0.08 mg H_2O_2/dL. Intra- and inter-assay CV were assessed at 3.22% and 8.19%, respectively.

Serum was extracted from a portion of the plasma samples by defibrination (Johnstone and Thorpe 1987) and serum antioxidant capacity was estimated with the OXY-Adsorbent Test (Diacron International) (Trotti *et al.* 2001). This test exploits the capacity of a massive solution of HClO to oxidise the complete

pool of antioxidants in serum. Thus, SAC considers the cumulative action of all the antioxidants present in serum, rather than simply the sum of measurable antioxidants. Results were expressed as mmol HClO/mL. Intra- and inter-assay CV were assessed at 2.85% and 5.10%, respectively. The OSi was calculated as ROS/SAC.

Analysis of plasma Cu, Se, BHOB concentrations

After completion of the assays for white blood cell function and oxidative status, the residuals from all heparin plasma samples were centrifuged at 2,000 g for 3 minutes and stored at 2–8°C. For samples collected at Visit 2, the RAND function in Excel was used to select, from each farm, 20 samples from each treatment group from which 1 mL was removed and sent to Awanui Veterinary (Christchurch, NZ). Plasma Cu and Se were measured by atomic absorption spectrophotometry (Thompson and Blanchflower 1971). Quality control measures indicated that the CV between sample runs was 8.3% for Cu (range within runs 4.6–9.2%), and 5.2% (range within runs 2.8–5.1%) for Se. BHOB concentrations were measured in mmol/L using colourimetric enzymatic reactions (Randox Laboratories Ltd., Ardmore, UK) according to the manufacturer's instructions and as described by Jahani-Moghadam *et al*. (2015).

Subsequently, for the samples from Visits 3, 4 and 5 (Figure 1), from each farm 15/20 of the animals from each group identified at the second visit for Cu, Se and BHOB testing were selected for repeat testing for Cu, Se and BHOB, with the aim being to ensure a minimum per farm of 10 complete sets/group of results containing Cu, Se and BHOB from the same cows at Visits 2, 3, 4 and 5.

Analysis of white blood cell population

Samples preserved with EDTA were transported overnight to Awanui Veterinary Laboratory (Christchurch, NZ) for assessment of WBCC. Total WBCC and differential counts were initially measured using a haematology analyser (Sysmex XN 1000; Sysmex, Kobe, Japan). Subsequently, a Leishman's-stained blood smear was prepared from each sample, the leucocytes were checked for normal morphology and the absence of lysis and a manual differential percentage for the different leukocyte types was recorded by counting a total of 100 cells on the slide. For the purposes of the study, leukocyte types other than neutrophils, lymphocytes and monocytes were combined into a single "other" category. Total WBCC (x 10^9 L), the manually derived percentage of each cell type and, as their product, the number of each cell type were reported (Herman *et al.* 2018). Major discrepancies between the automated and manually derived differential estimates led to a repeat of the manual estimation process to confirm normal cellular morphology.

Analysis of whole blood GPx activity

After completion of the assays for white blood cell population, the residuals from these samples were stored at −15 to −18°C. At Visits 2–5, samples were selected for GPx analysis using a similar procedure to that described for selection for Cu, Se and BHOB. GPx activity was measured using the methods of Paglia and Valentine (1967) and expressed in kU/L determined at a reaction temperature of 25°C.

Assessment of heat stress

Climate data was downloaded from the nearest weather station registered with the National Institute of Water and Atmospheric Research[1] (Timaru Aerodrome, latitude −44.31, longitude 171.25) and the unitless daily temperature humidity index (THI) was calculated according to the formula of Bryant *et al.* (2007).

Statistical analysis

All analysis was carried out using R v.4.2.1 (R Development Core Team; R Foundation for Statistical Computing, Vienna, Austria). The dependent variables (plasma Cu, Se and BHOB concentrations, whole blood GPx activity, fluorescence intensity, ROS, SAC, OSi, total WBCC, percentage of neutrophils and monocytes showing phagocytosis and the percentage of neutrophils, lymphocytes, monocytes and other white blood cell types) were analysed using Bayesian multivariable mixed models and the brms package (Bürkner 2017), with all models running with four Monte Carlo Markov chains and a total sample size of 16,000, of which 4,000 iterations were used for warm-up. We expected that the changes observed would be small, and where small changes are anticipated, Bayesian estimation can be useful as it involves the quantification of the probability of the outcome rather than the falsification of a null hypothesis. Further, prior knowledge about the likely distribution and range of variables could be incorporated into current estimation of parameter values, so reducing the probability of type 1 errors.

Within the data, responses were potentially clustered within individuals, within visits and within farms. Moreover, there were repeat responses from the same individuals measured on four occasions and the potential for the response to treatment to vary between farms and between visits. Different modelling structures were compared for each dependent variable including random intercepts for individual, farm, visit and random slopes for the effect of

[1]https://cliflo.niwa.co.nz/ accessed 13 November 2023

treatment and modelling farm, visit and treatment as fixed effects with and without interactions. Different approaches to model the covariance-variance structures in the data were also compared by comparing models with the default compound symmetry (equal covariance and variance for all random effects) with models using AR1 autocorrelation. The model's widely applicable information criterion (WAIC) value (Watanabe 2010), simplicity and the limited nature of the data (only four repeat measures and two farms) guided final model choice.

The primary aim was to assess the effect of TMS while adjusting for the effects of time and other covariates. Consequently, the model outputs were expressed in terms of the median predicted difference (and the 90 or 95% highest density interval (HDI); Kruschke 2018; Makowski *et al.* 2019), between treated and control cows for an average cow for each visit and for an average cow averaged over all visits following injection. Predictions were made only over the range of values for the predictor variables found within the current dataset. Predictions for the effect of TMS on white blood cell differentials were made both in terms of the number and the percentage of each cell count, and differences were expressed as the numeric difference in cell types, the difference in percentages of cell types, and as the relative numeric difference (numeric difference as a percentage of the control) for each cell type. Two samples were considered to be equivalent if the 95% probability distribution for the difference fell within a pre-defined margin of zero (the region of probable equivalence (ROPE); Kruschke 2015). This margin was defined as ± 0.5 years for cow age and ± 5% for all other numeric variables.

Full details of variable selection, model construction and assessment and pre-study power calculations are given in Supplementary Information.

Results

Multivariable models: variable selection and model structure

At enrolment, the mean age was 6.0 (SD 1.8) years, and 89% of enrolled cows were classified as Friesian (estimated body weight > 450 to < 550 kg) and when treated received a dose volume of 5 mL TMS, while 11% were classified as Friesian-Jersey cross (estimated body weight > 350 to < 450 kg) and when treated received a dose volume of 4 mL TMS. There was no evidence for confounding by age or breed at enrolment with a median difference in age between treatment groups of −0.12 (95% HDI = −0.73 to 0.51) years and a difference in the percentage of crossbreds between treatment groups of −0.2 (95% HDI = −7 to 7)%. Although the estimated HDI exceeded the ROPE (± 0.5 years and ± 5%, respectively), both were centred near the null value. Inclusion of age or breed did not improve the WAIC of the models over the period of the study.

In all models, modelling farm, visit and treatment and their three-way interactions as fixed effects resulted in lower WAIC values and allowed the effect of treatment to vary between farm and visit. Similarly, models where the covariance-variance structures in the data were modelled with a compound symmetrical structure resulted in lower WAIC values, faster model convergence and identical coefficient values to models with more complex autocorrelation structures. Coefficient values from all models were robust to changes in prior specification.

Missing data

At Visit 2, 2–4 weeks before PSC, a convenience sample of 240 of the 300 cows (from both farms) identified for potential enrolment were successfully enrolled (target 240). At Visit 3 (first visit after calving), 197 (target 200) of these were identified and sampled as having calved within 1 week of the herd's PSC. After resampling at Visits 4 and 5, complete records (all necessary tests required for each cow at all visits) were available for 171 cows for ROS, SAC, OSi, fluorescence and phagocytosis, while 168 cows had complete results for WBCC and differential.

Contemporaneously, at the same visit (Visit 2), 80 (target 80) of the sampled cows were randomly assigned for assays of plasma Cu, Se, BHOB and whole blood GPx. Subsequently, at Visit 3, samples from 60 (target 60) of these 80 cows were reanalysed for plasma Cu, Se, BHOB and whole blood GPx, and after resampling at Visits 4 and 5, complete records were available for 54 (target 60) cows.

The predicted difference in the mean age between cows with missing values and those with complete datasets was 0.13 (95% HDI = −0.28 to 0.50) years. The predicted difference in the percentage of complete datasets between the TMS and control group was 1.6 (95% HDI = −9.0 to 13.0)%; between farms it was 4.0 (95% HDI = −6.0 to 15.1)% and between breeds it was 0.4 (95% HDI = −17.7 to 17.5)%. Visual inspection of the pattern of missing data revealed a mix of completeness, but with the majority of missing cases lacking results for Visit 2 and subsequent visits, compatible with the aim of resampling the same cows at Visits 2–5. All missing datasets had complete sets of values for the independent variables but were lacking values for the dependent "y" variable. Consequently, data imputation methods were not attempted (Jakobsen *et al.* 2017).

Days calved at each visit

Farm visit dates were based on predicted calving dates and were designed to sample cows with a narrow

range of days in milk that was distinct and non-overlapping for each visit, without interfering with normal farm operations from multiple visits. Visits 1, 2, 4 and 5 were carried out as single visits for each farm, while for Visit 3, two visits were required for one farm and three for the other, to collect sufficient samples. The range of days in milk of enrolled cows is shown in Supplementary Table 2. Allowing for the range of dates over which cows were allocated to treatment or control (Visit 2) and the range of dates over which the visit centred at calving (Visit 3) took place, cows were at 258–273 days gestation at the time of TMS injection.

There was no difference between the calving dates of the TMS and control groups (mean calving date for TMS-treated and control cows was 5 August 2022 with a 95% HDI from 4 August 2022 to 5 August). There was no difference between treatment groups in the days relative to calving at any of the farm visit dates after calving. For both groups, the mean days calved at Visits 3, 4 and 5 were 3 (95% HDI = 2–4), 12 (95% HDI = 11–12), and 40 (95% HDI = 39–40) days, respectively.

Dependent variables where there was evidence for a difference from TMS

White blood cell differential

At Visit 2, 14–28 days before PSC and preceding allocation to treatment groups, the median number of neutrophils, lymphocytes, monocytes and other white blood cells was 2.9 (min 1.2, max 4.8), 2.9 (min 1.2, max 49), 0.4 (min 0.1, max 1.4) and 0.5 (min 0.1, max 2.2) x 10^9 cells/L, respectively, and the percentage of neutrophils, lymphocytes, monocytes and other white blood cells was 43 (min 18, max 71), 44 (min 18, max 72), 6 (min 1, max 20) and 7 (min 1, max 32)%, respectively. There was no biologically meaningful difference in the number or percentage of neutrophils, lymphocytes, monocytes or other white blood cell types between samples from cows allocated to the control or TMS groups (Supplementary Table 3).

While the number and percentages of neutrophils and lymphocytes within the WBCC remained within the reference ranges, there were differences following injection between samples from TMS-treated and control cows in the number and percentage of the WBCC comprising lymphocytes and neutrophils over time. After calving, the number and percentage of neutrophils in the WBCC from TMS-treated cows was less than from control cows.

At Visit 3, 3 (min 0, max 9) days after calving, and at Visit 5, 40 (min 36, max 45) days after calving, the certainty that this difference excluded zero was ≥ 90% with, respectively, 0.36 (90% HDI = 0.0–0.77) x 10^9 and 0.25 (90% HDI = 0.00–0.55) x 10^9 fewer neutrophils/L in TMS-treated cows. In terms of the composition of the white blood cell population, at 3 and 40 days after calving, in TMS-treated cows, neutrophils comprised 6 (90% HDI = 0–11)% and 4 (90% HDI = 0–8)% less of the total WBCC and the neutrophil count in TMS-treated cows was 14 (90% HDI = 0–27)% and 9 (90% HDI = 0–18)% less than in comparable control cows.

Conversely the number and percentage of lymphocytes in the WBCC was greater in samples from TMS-treated cows. At Visit 4, 12 (min 8, max 18) days after calving, and at Visit 5, 40 (min 36, max 45) days after calving, the certainty that this difference exceeded zero was ≥ 95% with, respectively, 0.65 (95% HDI = 0.17–1.17) x 10^9 and 0.28 (95% HDI = 0.00–0.59) x 10^9 more lymphocytes/L in TMS-treated cows. At 12 and 40 days after calving, in TMS-treated cows, lymphocytes comprised 10 (95% HDI = 3–18)% and 5 (95% HDI = 0–9)% more of the total WBCC and the lymphocyte count was 30 (95% HDI = 11–51)% and 9 (95% HDI = 0–9)% greater than control cows.

However, the effects were small and transitory such that there was too much uncertainty to infer a net effect over the entire period of follow-up. The model predicted that overall, in TMS-treated cows, the neutrophil count (2.7 (95% HDI = 1.7–3.7) x 10^9 cells/L) was 8% lower than in control cows (2.9 (95% HDI = 2.0–3.9) x 10^9 cells /L) but the 95% HDI for the difference extended from 28% lower to 7% higher. Similarly, the model predicted that in TMS-treated cows the lymphocyte count (2.9 (95% HDI = 2.0–3.9) x 10^9 cells/L) would be 8% greater than in control cows (2.7 (95% HDI = 1.7–3.7) x 10^9 cells/L) but with a 95% HDI extending from 8% lower to 36% higher.

The percentage of the WBCC comprising neutrophils and lymphocytes in samples from TMS-treated and control cows is shown in Figure 2.

Over the period of the study, the number of monocytes and other white blood cell types were always within the reference range. There were no meaningful changes in the percentages of the WBCC comprising monocytes nor other white blood cell types over time and the differences between the TMS and control groups were within a ROPE of ± 5%.

Percentage of neutrophils and monocytes showing phagocytosis and fluorescence intensity

At enrolment (Visit 2) the mean percentage of neutrophils and monocytes expressing phagocytosis was 27 (SD 14.5)%. However, 4 (95% HDI = 1.6–6.7)% more neutrophils and monocytes were expressing phagocytosis in the cows in the control group compared to cows in the treatment group. After calving, there was an increase for both groups in the percentage of neutrophils and monocytes expressing phagocytosis but at Visit 3, 3 (min 0, max 9) days after calving, the percentage of cells expressing phagocytosis was 7 (95% HDI = 2.1–12.3)% greater in cows treated with TMS.

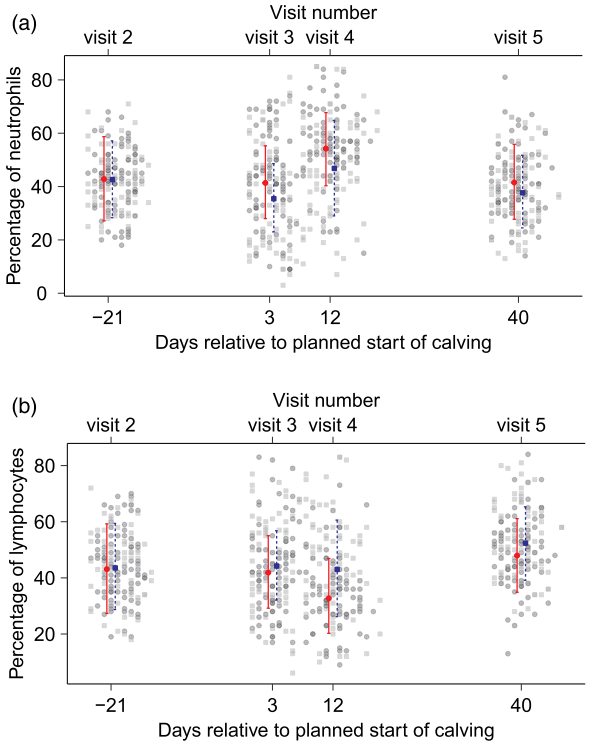

Figure 2. Percentage of (a) neutrophils and (b) lymphocytes in the blood of periparturient cows following SC injection at Visit 2 with either hydroxocobalamin and Se (controls: grey circles) or with hydroxocobalamin and a trace mineral supplement containing Zn, Mn, Se and Cu (TMS: grey squares). Bayesian, mixed model predictions for the most likely marginal value for cows in the control group (solid red circles) and the TMS group (solid blue squares) are shown with the 95% highest density interval shown as error bars. A colour version of this figure is available online at the URL provided in the article header.

At Visits 4 and 5, 90% of the most likely predicted differences between treatment and control groups fell within the ROPE of ± 5% and so indicated with 90% certainty that phagocytosis was equivalent between TMS and control cows at these visits (Figure 3a). Over the entire follow-up period, and allowing for the initial difference in phagocytosis between samples from TMS-treated and control cows, the model predicted that the total difference in phagocytosis attributable just to TMS injection was 4.5 (95% HDI = −2.7 to 13.8)% greater in the samples from TMS-treated cows compared to control cows.

At enrolment (Visit 2), the median plasma fluorescence intensity was 5,119 (min 570, max 19,350) MESF. The model predicted that at enrolment there was > 90% probability that fluorescence was higher in the samples from TMS-treated cows than from the control cows (difference = 1,036 (90% HDI = 57–2,332) MESF). After calving, fluorescence decreased in both TMS and control groups but at Visit 3, 3 (range min 0, max 9) days after calving, the model predicted with a 90% probability that fluorescence was still higher in the samples from TMS-treated than control cows (difference = 424 (90% HDI = 5–814) MESF). Thereafter, although the most likely value for the model's estimate of fluorescence remained numerically higher for samples from TMS-treated cows (Figure 3b), the range of the 90% most likely values for the difference included negative as well as positive values (difference at Visit 4 = 304 (90% HDI = −730 to 1,286) MESF and at Visit 5, difference = 279 (90% HDI = −707 to 2,013) MESF). Over the entire period of follow-up and allowing for the initial difference in fluorescence between samples from TMS-treated and control cows, the model predicted that the total difference in fluorescence attributable just to TMS injection was 1,980 (95% HDI = 1,563–2,190) MESF greater in the samples from TMS-treated cows compared to control cows.

Dependent variables where there was evidence for no difference from TMS

Plasma Cu, Se, BHOB and whole blood GPx

At the end of the preceding lactation at Visit 1, all 15 animals on both farms had concentrations of Cu, and Se and activity of GPx in plasma above the marginal range (9 µmol/L for plasma Cu (Laven *et al.* 2007);

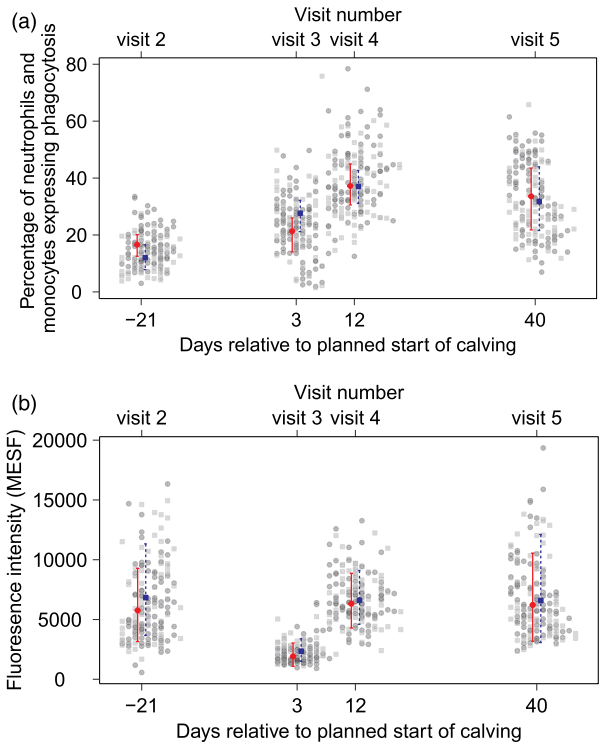

Figure 3. Phagocytic activity of neutrophils and monocytes showing in the blood of periparturient cows following SC injection at Visit 2 with either hydroxocobalamin and Se (controls: grey circles) or with hydroxocobalamin and a trace mineral supplement containing Zn, Mn, Se and Cu (TMS: grey squares) as measured by (a) percentage showing phagocytosis and (b) fluorescence intensity. Bayesian, mixed model predictions for the most likely marginal value for cows in the control group (solid red circles) and the TMS group (solid blue squares) are shown with the 95% highest density interval shown as error bars. A colour version of this figure is available online at the URL provided in the article header.

250 nmol/L for whole blood Se and 2 kU/L for GPx (Hendriks and Laven 2020)).

Subsequently over Visits 2–5, Cu, Se and GPx remained above these published marginal limits and the plasma concentration of BHOB remained below the cut-off of ≥ 1.4 mmol/L for subclinical ketosis (Oetzel 2004). There was effectively no difference in concentrations by treatment group (Supplementary Table 3), with the models predicting that the 95% most likely value for the difference in Cu, Se, GPx and BHOB between the TMS and control groups was < 5% of the mean respective value.

Oxidative status: ROS

At the second visit when the cows were enrolled into their treatment groups, the median ROS was 178 (min 87, max 312) CarrU. There was no evidence for a difference in ROS between treatment groups at enrolment, although the 95% HDI exceeded the ROPE of ± 5% so equivalence could not be assumed (Supplementary Table 3). Over calving, the level of ROS briefly increased in both treatment groups (Supplementary Figure 1) but the differences between groups in the level of ROS were small.

Over all visits, control animals were predicted to have a ROS of 180 (95% HDI = 139.4–215.6) CarrU and TMS-treated cows 185 (95% HDI = 152.5–230.3) CarrU with a predicted difference in ROS of 5.1 (95% HDI = −13.7 to 34.3) CarrU. This was larger than the ROPE of ± 5% and so we cannot assume there is no difference between the two groups as some of the most likely values fall outside the ROPE (Kruschke 2015).

Oxidative status: SAC

At Visit 2, when the cows were enrolled into their treatment groups, the mean SAC was 337 (SD 108) mmol HClO/mL. There was no evidence for a difference in SAC between treatment groups, although the 95% HDI of the difference again exceeded the ROPE of ± 5% (Supplementary Table 2). Over calving the level of SAC remained virtually constant in both treatment groups (Supplementary Figure 2) and the differences between groups in the level of SAC were small.

Over all visits, control animals were predicted to have a SAC of 332 (95% HDI = 260.4–405.3) HClO/mL and TMS cows 338 (95% HDI = 249.0–421.7) HClO/mL with a predicted difference in SAC of 4.8 (95% HDI = −26.9 to 32.4) HClO/mL. Although this was larger than the ROPE of ± 5% and some of the most likely values fall outside the ROPE, the symmetry of the 95% HDI suggests that this was because of scatter in the data and a larger sample size may well have consolidated the predicted differences to be within the ROPE.

Oxidative status: OSi

At Visit 2, when the cows were enrolled into their treatment groups, the median OSi was 0.54 (min 0.19, max 17.67) and the OSi ratio was equivalent for both treatment groups (Supplementary Table 2). Over calving, the OSi peaked at calving (corresponding to the increase in ROS and a slight decrease in SAC) but the differences between treatment groups remained small (Supplementary Figure 3).

Over all visits, control animals were predicted to have an OSi of 0.75 (95% HDI = 0.35–1.03) and TMS-treated cows 0.70 (95% HDI = 0.41–1.01). At all times and for both groups, the predicted OSi and the 95% HDI were ≤ 1.0 suggesting that the ROS exceeded the SAC. There was no evidence for a difference in the OSi between control and TMS-treated cows with, overall, the OSi for control cows being 0.98 (95% HDI = 0.70–1.44) times the OSi for TMS-treated cows.

Total white blood cell count

At Visit 2 the median WBCC was 7 (min 2, max 17) x 10^9 cells/L compared to a reference interval of 4–10×10^9 cells/L. Over all visits, control animals were predicted to have a WBCC of 6.5 (95% HDI = 4.3–9.1) x 10^9 cells/L and TMS-treated cows 6.5 (95% HDI = 4.2–9.0) x 10^9 cells/L with an overall predicted difference in the WBCC between samples from TMS-treated and control cows of −0.05 (95% HDI = −1.2 to −1.0) x 10^9 cells/L. At all times and for both groups, the predicted counts were within the reference range and although the 95% HDI for the differences by visit and overall were greater than ± 5% of the median count, the predicted counts did not exceed the reference range and differences were all centred equally around zero (Supplementary Table 3 and Supplementary Figure 4).

Heat stress

The median 3-day average THI over the period of the study was 54.7 (min 46.6, max 63.2) and at no point did the THI approach the levels independently identified by Bryant *et al*. (2007) and Silva *et al*. (2022) as consistent with heat stress sufficient to reduce production (68 for Holstein-Friesian).

Discussion

This study reports small changes in white blood cell population and function after calving in cows receiving injectable TMS containing Zn, Mn, Se and Cu 14–28 days before calving. After calving, the neutrophil count in treated cows was 14 (90% HDI = 0–27)% less than controls, while the lymphocyte count was 30 (95% HDI = 11–51)% greater. This effect could still be detected 40 days after calving with the neutrophil count in treated cows 9 (90% HDI = 0–18)% less than

controls, while the lymphocyte count was 9 (95% HDI = 0–9)% greater.

Phagocytosis was also increased in cows receiving TMS with 7 (95% HDI = 2.1–12.3)% more white blood cells expressing phagocytosis 3 days after calving and an increase in bacteria ingested per white blood cell over the 40 days of follow-up.

Although Machado *et al*. (2014) found no improvement 10 days after calving in white blood cell function in cows receiving TMS at 230 and 260 days of gestation, the direction and magnitude of the changes reported in the current study are broadly consistent with the response to TMS injection reported by other researchers (Teixeira *et al*. 2014; Bates *et al*. 2022; Silva *et al*. 2022). However, the measures of immunity and the effects of TMS on immunity were more varied in the present study than in these studies. In adult dairy cows given two injections of TMS at 208 and 260 ± 3 days of gestation, Silva *et al*. (2022) reported a similar magnitude of increase in the percentage of cells expressing phagocytosis, although the effect persisted for more than 35 days after injection. However, in that study, the mean value of the percentage of cells expressing phagocytosis was greater and the variance in the number of cells expressing phagocytosis was less than in the present study. Although differences in experimental and analytical methods and potential breaches in storage conditions during transport make direct comparison between studies difficult, the greater variance and lower mean values for variables describing immune function in pastoral dairy cows is intriguing and warrants further investigation.

Data from studies reporting the effect of TMS in dairy calves also indicate a similar increase in phagocytosis. Bates *et al*. (2020) reported that the white blood cells from neonatal calves on a New Zealand pastoral dairy farm receiving TMS showed a 15.4 (95% HDI = 10–20.4)% increase in phagocytosis 1 week after injection and a 7.5 (95% HDI = 2.1–13.7)% increase after 2 weeks, and a persistent increase in the number of bacteria ingested per cell similar to the present study. A similar effect from TMS injection was also reported for neonatal calves in an intensive, housed dairy system by Teixeira *et al*. (2014). Both studies indicated similar levels of phagocytosis in the enrolled calves, and these were comparable to those reported by Silva *et al*. (2022) in adult cows and higher than the results reported here.

After calving, cows in the control group experienced a mild increase in the neutrophil count and a mild decrease in the lymphocyte count similar to that reported by Meglia *et al*. (2001), although in the current study, both effects were observed later (12 days) than reported by Meglia *et al*. Compared to control cows, TMS appeared to reduce both these effects, with a smaller increase in the neutrophil count in TMS-treated cows and a small increase in the lymphocyte count, consistent with the effect of TMS in decreasing the neutrophil count and increasing the lymphocyte count and the decrease in the neutrophil to lymphocyte ratio in treated cows. We observed no changes in the total WBCC following TMS injection and this finding was also reported by Machado *et al*. (2014) and Silva *et al*. (2022) following two injections of TMS in the last 60 days of the dry period. However, these authors also reported no change in the neutrophil to lymphocyte ratio. We do not know whether the temporary reduction in neutrophil count in cows receiving TMS in the current study was a result of migration of neutrophils into tissues such as the udder and uterus, nor the implications of the lower neutrophil to lymphocyte ratio.

The inconsistencies in the effect of TMS between studies highlight some of the limitations of the current work. Under New Zealand's pastoral and seasonal management system, dry cows are often wintered at grazing properties with minimal handling facilities, and management interventions during the dry period can be difficult. Consequently, we chose to inject dry cows with TMS only once and at a time when they were already scheduled to be drafted. We do not know what effect the co-injection with a vaccine may have had on the response to TMS, nor whether the changes in white blood cell population would have been more similar to those seen in other studies if more doses of TMS had been given. Equally, the sample size of the current study was not designed to be sufficient to assess the clinical impact of the changes in white blood cell population and function reported in this study. However, reductions in clinical and subclinical mastitis have been reported in a similar study involving more cows (Bates *et al*. 2022) and in studies where more doses of TMS were administered (Machado *et al*. 2013; Silva *et al*. 2022).

Although multiple studies have now been published suggesting that TMS injection can reduce morbidity and leads to changes in the immune response, we are very far from understanding how these events are related. In this analysis, all coefficient values were robust to changes in the priors used, indicating that the estimation of the coefficients was driven by the data. Thus, although the effects are small, the models indicate that there is a high degree of probability that the effects exclude zero.

Intuitively, if TMS increases phagocytic capability this appears to be consistent with a reduction in disease and Cebra *et al*. (2003) found that serum Se concentrations were positive predictors of neutrophil function in periparturient dairy cows. However, in that study, the serum concentrations of Se were 4–5 times higher than in the current study so the same association cannot be assumed to be present in this case.

In the present study, all animals were receiving mineralised drinking water and had been injected

with a comparable dose of Se and hydroxycobalamine at enrolment. In this situation, all animals had plasma mineral and BHOB concentrations consistently within the reference ranges and there was no effective difference in the plasma concentration of Cu, Se, and BHOB or activity of GPx between the TMS and control groups. This suggests that the effects on white blood cell function and population cannot be replicated simply from the administration of Se rather than from the combination of minerals in the TMS. Both Machado *et al*. (2014) and Silva *et al*. (2022) reported a lack of effect of TMS supplementation on serum GPx activity. However, in the study by Machado *et al*. (2014), BHOB was marginally lower in older cows receiving TMS, although in both groups it was consistently below the concentration indicating subclinical ketosis.

The greater variance in WBCC in the present study, the lower percentage of white blood cells expressing phagocytosis and the delay in the decrease in neutrophil count seen after calving compared to studies from non-pastoral systems suggest that the efficacy of TMS may vary between systems and stages of lactation and that in pastoral systems predicting which cows will benefit from TMS is difficult. This is consistent with the wider predictive intervals for the effect of TMS on clinical and subclinical mastitis reported in these systems by Bates *et al*. (2020). There was no evidence in the present study that the THI was high enough to be precipitating heat stress, which has been suggested to increase the concentration of ROS (Rhoads *et al*. 2013), and the reasons for the variability in the effect of TMS remain unclear. However, given the focus on antimicrobial reduction and maximising efficiency of production to offset greenhouse gas emissions, TMS appears to be an effective mechanism for some cows to improve the innate immune system and reduce disease. Further work is required to identify the cow- and farm-level factors that will identify the optimum animals for treatment.

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AJ Bates is the Scientific Editor for the *New Zealand Veterinary Journal*.

ORCID

AJ Bates http://orcid.org/0000-0003-0732-4262
M Wells http://orcid.org/0009-0002-5893-2604
C Fitzpatrick http://orcid.org/0000-0001-9946-9252
RA Laven http://orcid.org/0000-0002-8938-8595

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